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Biochemical and Biophysical Research Communications 362 (2007) 568–574
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Activin B mediated induction of *Pdx1* in human embryonic stem cell derived embryoid bodies

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Received 24 July 2007
Available online 15 August 2007

Abstract

Human embryonic stem cells (hESCs) have the potential to provide alternative sources for pancreatic islet grafts. In the present study we have investigated the influence of Activin A and Activin B on the expression of the pancreas marker gene *Pdx1* in hESCs differentiated as embryoid bodies (EBs). We report here that Activin B in a dose depend manner markedly up-regulates *Pdx1* expression as compared to Activin A and untreated cultures. *Pdx1*(+) cells co-express *FOXA2* but lacks, however, co-expression with *nkx6.1*, a marker combination that in the present study is shown precisely to identify embryonic and fetal pancreas anlage in humans. *Pdx1*(+) cells are found in cell clusters also expressing *Serpina1* and *FABP1*, suggesting activation of intestinal/liver developmental programs. Moreover, Activin B up-regulates Sonic Hedgehog (*Shh*) and its target *Gli1*, which during normal development is suppressed in the pancreatic anlage. In conclusion, Activin B is a potent inducer of *Pdx1* as well as *Shh* in differentiating hESCs. The data suggest that additional suppression of *Shh* signaling may be required to allow for proper specification of pancreatic cell lineages in hESCs.

Keywords: Activin; Embryonic stem cells; Hedgehog; Pancreas development

Success of cell-replacement therapy for diabetes may depend on the establishment of alternative sources of pancreatic islet grafts. hESC differentiation toward pancreatic insulin-producing cells offers such perspectives, but there are still many challenges to overcome. A better understanding of the extra-cellular signaling required for the proper development of pancreatic cell lineages is one important issue. Pancreatic cells differentiate from definitive endoderm-derived progenitor cells. The definitive endoderm portion fated to become pancreas receives signals from adjacent layers and becomes responsive to pancreas-inducing cues. In mammals, notochord, aortic endothelium, and neighboring mesenchymal cells coordinate pancreas specification within the competent endoderm. Signals from these tissues initiate a transcription factor network which eventually results in the specification of a *Pdx1*(+) pancreas precursor population that will further differentiate into exocrine, endocrine, and duct cells [1–6]. Among these signals Activins, which belong to the TGFβ superfamily, play a prominent role. Activins are dimers of distinct subunits (βA and βB). Activin A (βA/βA), Activin B (βB/βB) and Activin AB (βA/βB) share the four activin receptors ActRI/ALK2, ActRIB/ALK4, ActRII, and ActRIIB [7]. The Activins bind to heterodimeric receptors composed of a type II and a type I subunit. The type II subunit is required for ligand binding and it phosphorylates and activates the type I subunit. Activated type I receptors interact with Smad proteins, which regulate the transcription of selected genes [7]. Because of the differential expression, in time and space, of the members of this signaling network, it is unclear to what extend specific Activin ligands control specific developmental events.

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doi:10.1016/j.bbrc.2007.07.200

Work in chick embryos showed that pancreas specification requires notochord-mediated repression of Sonic hedgehog (*Shh*) in the prepancreatic endoderm and that this repressive activity can be mimicked by Activin B [8]. Activin A is expressed with its receptors in the developing pancreas and ActRII$^{-/-}$ and ActRII$^{+/-}$ActRIIB$^{+/-}$ mice have impaired endocrine development [9]. It has, furthermore, been demonstrated that stimulation of Activin A signaling pathways is sufficient to induce *pdx1* expression in endoderm [10,11]. More recent experiments using mouse and human embryonic stem cells have also identified Activin A as being important for specification of definitive endoderm with a liver and pancreas developmental potential [12–17]. The aim of the present work was to reassess the possible influence of Activin A and Activin B on the specification of pancreatic endoderm in differentiating human embryonic stem cells. We demonstrate that Activin B is a potent inducer of *Pdx1* as well as *Shh* dependent signaling in differentiating hESCs. The data suggest that additional suppression of *Shh* signaling may be required to allow for proper pancreas specification in hESCs.

Materials and methods

Cell culturing and cell handling. The undifferentiated hESC lines HUES-9, was obtained from the Howard Hughes Medical Institute [18]. The Odense-3 hESC line was derived and characterized by the KMEB laboratory according to the procedures previously described [18], under the approval from the local Ethics Committee and with written informed consent by the donors. Handling and culturing of both hESC lines were performed as previously described [18].

Teratoma formation, kidney transplantation, and surgical procedures. Mice (NOD/SCID) were anesthetized and access to the left kidney was obtained by a lumbar incision. hESCs were aspirated into a polythene tube, spun down, and carefully injected under the kidney capsule. Subsequently the capsule was cauterized and carefully replaced. After 8 weeks the mice were sacrificed and the teratoma was isolated, fixed in 4% normal buffered formalin and embedded in paraffin.

Human embryo and fetuses. Human embryos and fetuses were collected at the local Department of Gynecology and Obstetrics and approved by the Regional Scientific Ethical Committee.

Real-time quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using the 6100 Nucleic Acid Prep Station (Applied Biosystems) and used for cDNA synthesis. Gene expression was analyzed using a custom-designed 384-well micro fluidic card (Applied Biosystems) using the ABI 7900HT Sequence Detection System (Applied Biosystems). Gene-expression level was normalized using the endogenous control gene *beta-actin* and the relative gene-expression level was determined using the 2-(-delta delta C_T) ($\Delta\Delta C_T$) method [19]. After normalization, the samples were plotted relative to the expression level in the control condition or in some case relative to the expression level in human pancreas. Data are presented as means from three independent experiments with the standard deviation.

Immunocytochemistry. Human EBs were collected at day 3, 5, 10, 15, and 20, fixed for 10 min in 4% formaldehyde and embedded in paraffin and sectioned (4 μm). Immunohistochemical staining was performed using DAKO En Vision+ and PowerVision. Primary antibodies was diluted in ChemMate Antibody diluent (S2022, DAKO); Oct-4 (SantaCruz (SC) (1 + 100), FOXA2 (SC, 1 + 200), HNF4alfa (SC, 1 + 200), Pdx1 (1 + 5000, R. Scharfmann), Nkx6.1 (DHSB, 1:2000). CK-18 (DAKO, 1 + 25), AFP (DAKO, 1 + 2000), Albumin (DAKO, 1 + 80.000), FABP1 (R&D, 1 + 500), Shh (SC, 1 + 100), Gli1 (SC, 1 + 100). Focal areas immunoreactive of 5 up to100 *pdx1*(+) cells were counted in 1000 EBs in all conditions ($n = 3$).

Western blotting. ES cells and EBs were washed in D-PBS and lysed in RIPA buffer supplemented with protease inhibitors. After 1-h incubation at 4 °C, samples were sonicated 3 × 20 s and centrifuged for 10 min (4 °C; 14,000 rpm). Protein concentration was determined with a BCA kit (Bio-Rad), and 50 μg were loaded on 10% polyacrylamide gel. Blotted nitrocellulose membranes were incubated overnight with anti-beta-actin antibody (Cell Signaling, 1 + 2500, 45 kDa) and anti-phospho Smad 2/3 antibody (Cell Signaling; 1 + 2500, 58 kDa). Signals were developed after binding of the secondary anti-rabbit horseradish peroxidase-labeled antibody (1/500; Santa Cruz Biotechnology) using ECL technology and Kodak films.

Results

Specification of pancreas endoderm in hESC derived teratomas

Histological analysis of hESC derived teratomas revealed focal areas resembling pancreas endoderm as demonstrated by the co-expression of the transcription factors *FOXA2*, *Pdx1,* and *nkx6.1*. Cells expressing glucagon or insulin were not observed (data not shown). These data demonstrate that pancreas like endoderm can be specified from the hESC cell lines Odense 3 and HUES-9. Representative data from transplanted HUES-9 cells are presented in Fig. 1A–F.

Activin B promote specification of cells expressing Pdx1

In order to investigate the potential differential effects of Activin A and Activin B, respectively, on the specification of pancreas endoderm in hESCs, we cultured embryoid bodies in the presence of Activin A or Activin B for up to 20 days. Samples for RT-PCR analysis were obtained at day 5, 10, 15, and 20. The results demonstrate that the transcription factor *Pdx1* required for normal pancreas development becomes up-regulated between day 15 and 20 of culture when exposed to Activin B as compared to Activin A and control treatments (Fig. 1). To further explore the potent action of Activin B on the specification of pancreatic endoderm, *Pdx1* protein expression were examined in EBs cultured for 20 days without Activin (Control), with Activin A (50 ng/ml), Activin B (50 ng/ml) and Activin B (50 ng/ml) together with SB431542 (10 μM), a specific Activin receptor antagonist. *Pdx1* immuno-reaction was observed in cell clusters containing approximately 5–100 cells in approximately 25% of the analyzed EBs when treated with Activin B, whereas very few *Pdx1*(+) cells were observed in the Activin A treated cultures and none were observed in the untreated control cultures or in cultures treated with Activin B and SB431542 (Fig. 1). To investigate a potential dose dependent effect of these observations, EBs were cultured for 20 days and exposed to different concentrations of Activin B or Activin A, respectively (0, 5, 10, 20, 50, and 100 ng/ml). Specific marker genes of the early pancreas endoderm (*FOXA2*, *Pdx1*, *Glucagon,* and *GATA-4*) were found to be up-regulated in a dose dependent manner by Activin B,

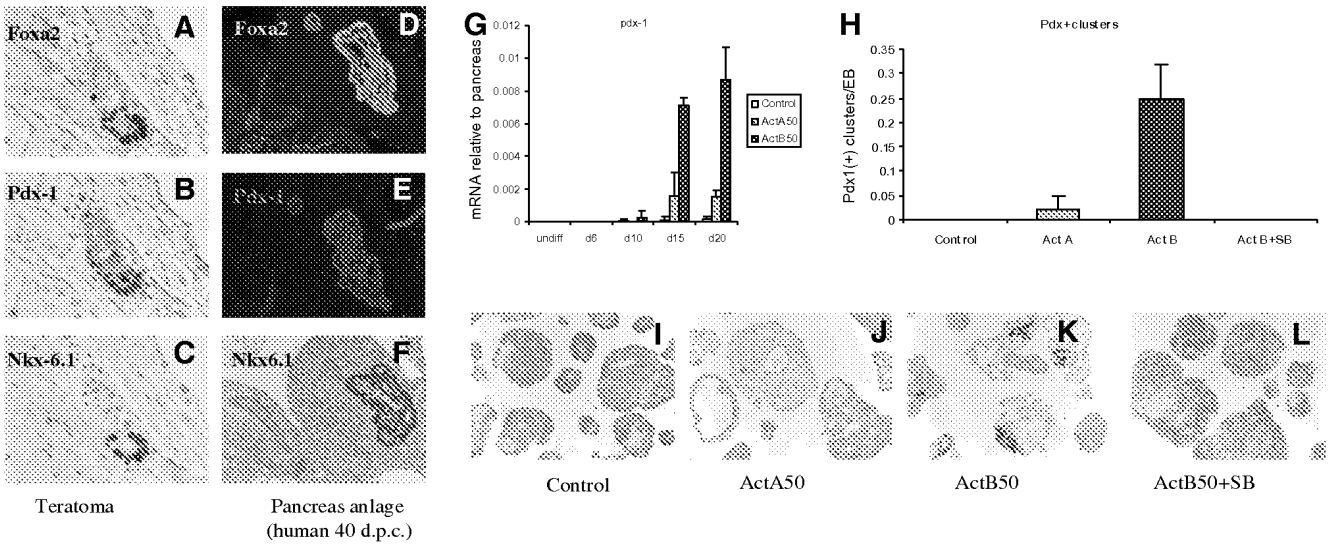

Fig. 1. Induction of *Pdx1* expressing cells in differentiated hESCs, *in vivo* and *in vitro*. (A–F) Immunoreactivity showing close similarity between pancreas-like anlage specified in hESC teratomas as compared to the pancreas anlage observed during normal pancreas development in humans. (A) *FOXA2* (B) *pdx1* (C) *nkx 6.1* in serial sections obtained from hESC derived teratomas. (D) *FOXA2* (E) *Pdx1* (F) *nkx 6.1* in serial sections obtained from a human embryo 40 d.p.c. (G–L) Activin B upregulate *Pdx-1* expression in hESC derived EBs. (G) Real-time PCR analysis of *Pdx1* in EBs cultured for 5, 10, 15, and 20 days without Activin (Control), with 50 ng/ml Activin A (ActA50) and with 50 ng/ml Activin B (ActB50), data are presented relative to pancreas control. (H) Graph showing the relative distribution of *Pdx1* immunoreactive EB's treated for 20 days without Activin (Control), with 50 ng/ml Activin A (ActA50) with 50 ng/ml Activin B (ActB50) and with 50 ng/ml Activin B and 10 μM SB431542 (ActB50 + SB). *Pdx1* immune reactivity in EBs treated 20 days (H) without Activin, (I) with 50 ng/ml Activin A, (J) with 50 ng/ml Activin B and (K) with 50 ng/ml Activin B and 10 μM SB431542. Note that Activin B induces formation of *Pdx1* immunoreactive cell clusters and that the induction is abolished by the Activin signaling antagonist SB 431542.

with the highest response at 50–100 ng/ml compared to Activin A and control cultures. Insulin and somatostatin gene expression was not detected (data not shown). Contrary, Activin A treatment was associated with a dose dependent up-regulation of *Oct-4/POU5F1* (Fig. 2) as well as *Nodal* and *Cripto* gene expression (data not shown), all genes associated with undifferentiated maintenance of hESCs. The Activin B induced *Pdx1* expressing cells co-express *FOXA2*, thus demonstrating the endodermal nature of these cells (Fig. 2). The induction of *Pdx1* as well as *FOXA2* positive cells was abolished by treatment with the Activin receptor antagonist SB-431542 and the developmental fate under these conditions was directed towards a neuro-ectodermal fate as demonstrated by the abundant expression of the pan-neuronal marker *TUJ-1* (Fig. 2G).

Both Activin A and Activin B were proven functional by their ability to induce phosphorylation of *SMAD2/3* (Fig. 2H and I) and by their ability to suppress the neuron-ectodermal marker gene *NeuroD1* (Fig. 2F), as previously described [20]. In summary, these data show that Activin A and B in a dose dependent manner promote differential developmental processes in hESC cells, were Activin A support self-renewal mechanisms and Activin B mediate differentiation processes as demonstrated by the induction of cells expressing *Pdx1*.

Co-localization of Pdx1 and nkx6.1 identify pancreas progenitor cells in humans in vivo

The transcription factor *Pdx1* is among the earliest proteins in both the ventral and dorsal rudiments and the transcription factor is considered one of the few markers that define the very early pancreatic anlage. In the mouse *Pdx1* is, however, also expressed in the adjacent duodenum [21,22]. More recent studies in mice have identified the transcription factor *nkx6.1* as an additional and more restricted marker of the pancreatic anlage [21,23]. To what extend this also applies during the development of the pancreatic anlage in humans is not known. Thus, in order to investigate this we gained asses to fixed human embryo material at 5–12 weeks post conception (w.p.c.). Immunohistochemial stainings of transverse section of these embryos revealed prominent *Pdx1* staining in the early developing pancreas as well the duodenum whereas the expression of *nkx6.1* was restricted to the pancreas epithelium (Fig. 3). The present data demonstrate that specific pancreatic progenitor cells can be identified in humans by the co-expression of *Pdx1* and *nkx6.1*. To identify whether hESC *Pdx1*(+) cells derived through Activin B treatment in the present study represent pancreas endoderm or alternatively a more intestinal/liver endoderm phenotype, we examined to what extend *nkx6.1* expression was found in *Pdx1* expressing cells. Immunohistochemical analysis revealed that none of the *Pdx1* (+) cells co-express *nkx6.1*, indicating that the *Pdx1*(+) cells generated by Activin B treatment does not represent pancreas progenitor cells (Fig. 3). The notion that the *Pdx1* is expressed in other endodermal cells types was supported by further immunohistochemical analysis showing co-expression with *FABP1*, *Serpina-1*, *E-cadherin*, *cytokeratin-18*, and *FOXA2* (Fig. 3).

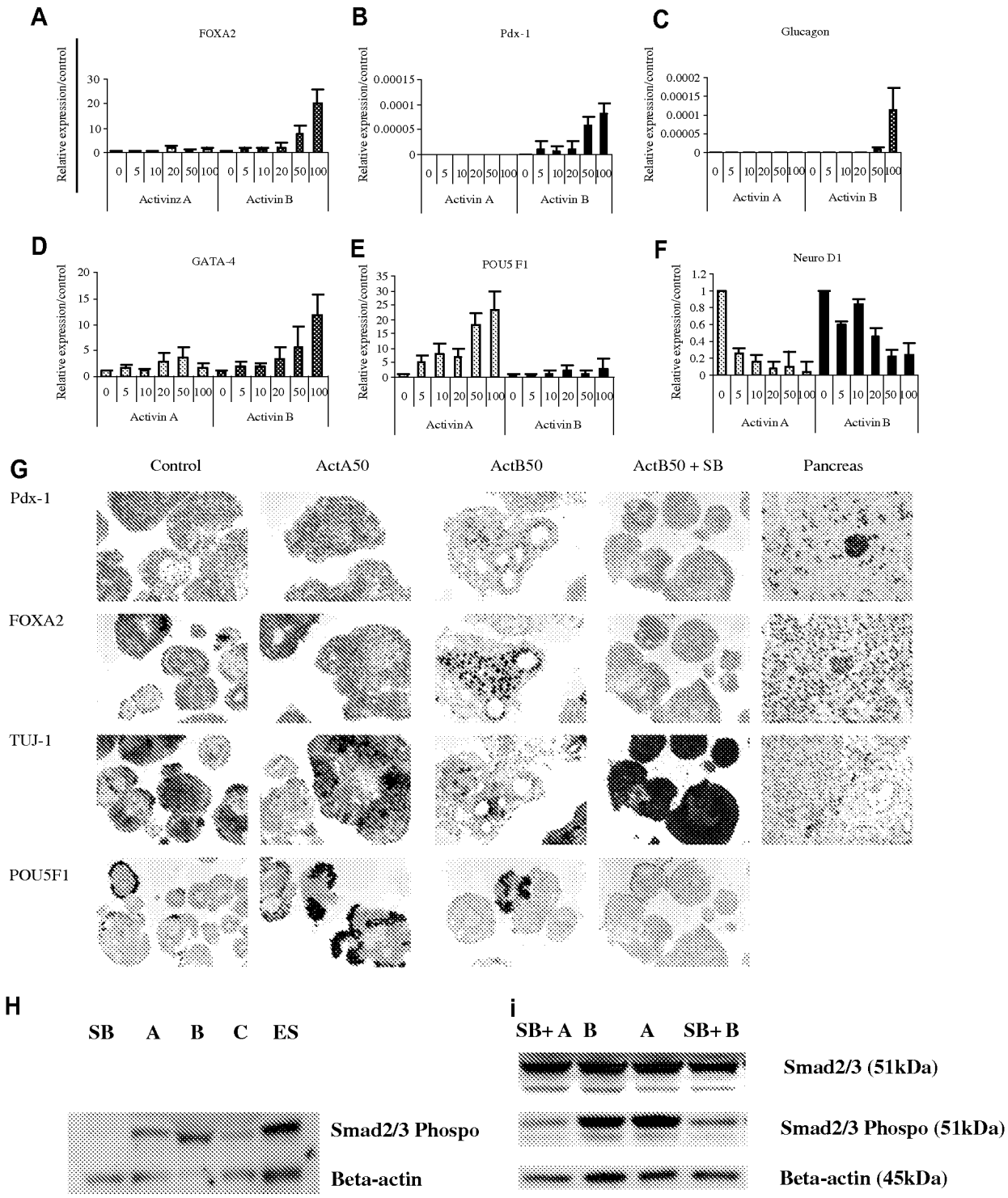

Fig. 2. Real-Time PCR analysis of EBs treated for 20 days without Activin or with 5, 10, 20, 50, and 100 ng/ml Activin A or Activin B. Activin B induces in a dose dependent manner up-regulation genes implicated in the early specification of the pancreas (A) *FOXA2*, (B) *Pdx1*, (C) *Glucagon*, and (D) *Gata-4* gene expression. (E) Activin A, on the other hand induce in a dose dependent manner *Oct-4/POU5F1* gene expression, which normally govern undifferentiated hESC self-renewal. (F) *NeuroD1* gene expression is as previously described down regulated by both Activin A and Activin B, thus demonstrating the functionality of both Activins. (G) Immuno-reactivity for *Pdx1*, *FOXA2*, *TUJ-1* and *Oct-4/POU5F1* in EBs treated for 20 days with without Activin (control), with 50 ng/ml Activin A (ActA50), with 50 ng/ml Activin B (ActB50) and with Activin B and 10 μM SB431542 (ActB50 + SB) compared to the immunoreaction observed in adult human pancreas. Note the abolishment of *FOXA2* and *Pdx1* expression in EBs treated with Activin B and SB431542 with a parallel up-regulation of the neuronal marker *TUJ-1*. (H,I) Phosphorylation of Smad2/3 in hESCs. (H) hESC derived EBs were grown for 72 h in the presence of EB media (C) or 50 ng/ml Activin A (A) or 50 ng/ml Activin B (B) or 10 μM SB431542 (SB) and compared to undifferentiated hESCs grown on MEF fedders (ES). The phosphorylated form of *Smad2/3* (*Smad2/3 Phospo*, 51 kDa) was detected on Western blots together with *Beta-actin* (Beta-actin, 45 kDa). (B) as in (A) however 10 μM SB431542 was added together with Activin A (SB + A) and Activin B (SB + B), respectively, to confirm the phospho specific actions of Activin A and Activin B, respectively.

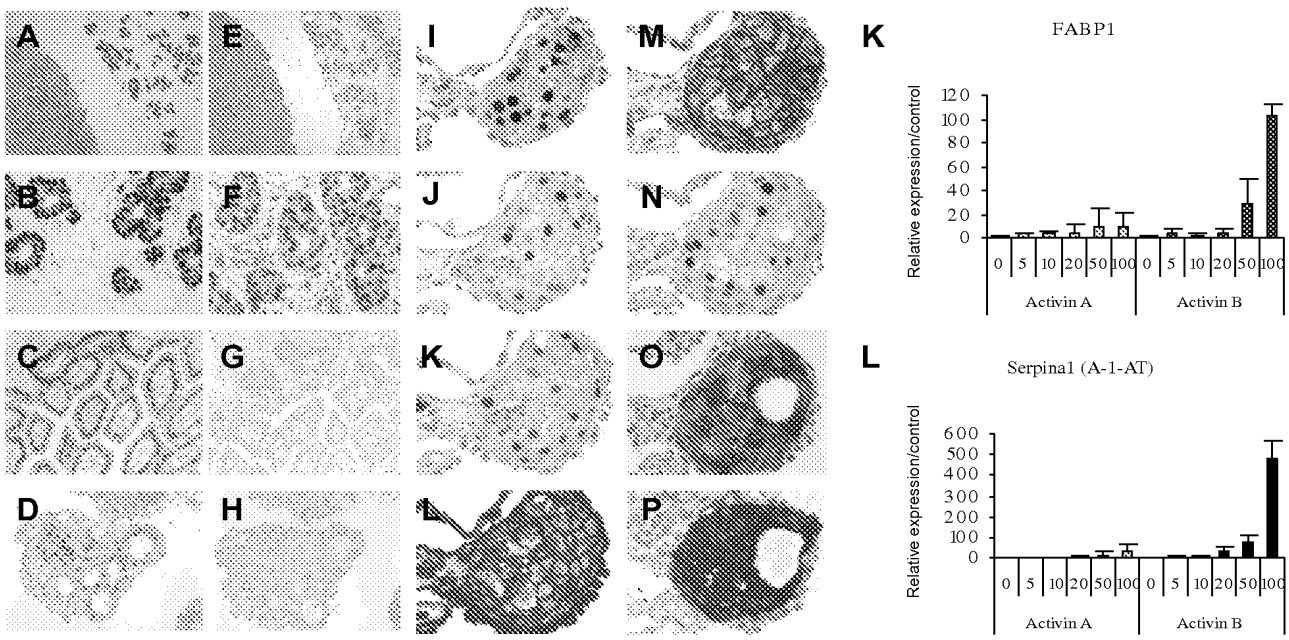

Fig. 3. Immunohistochemical stainings of human embryo 60 d.p.c. (A–H). (A,B) *Pdx1* expression in pancreas. (C) *Pdx1* expression in duodenum. (D) *Pdx-1* expression in Activin B treated EB day 20. (E,F) *Nkx6.1* expression in pancreas. (G) *Nkx6.1* expression in duodenum. (H) *Nkx6.1* expression in Activin B treated EBs day 20. The data show that the pancreas anlage in humans can be identified by the co-expression of *Nkx6.1* and *Pdx-1*. *Pdx1* immunoreactive cells found in EBs treated with Activin B does not co-express *Nkx6.1*, consequently these cells have not been committed to a pancreas developmental fate at this stage. (I–P) Immune histochemical stainings on serial sections of EBs treated with Activin B for 20 days; (J) *FOXA2*, (I) *Pdx-1*, (K) *GATA-4*, (L) *CK-18*, (M) *E-cadherin*, (N) *Pdx1*, (O) *Serpina-1*, (P) *FABP1*. The data show that *Pdx1* immunoreactive cells are found in cell clusters also expressing epithelial and intestinal/liver markers. (K,L) Real-time PCR analysis of EBs treated for 20 days without Activin or with 5, 10, 20, 50, and 100 ng/ml Activin A or Activin B. Activin B induces in a dose dependent manner up-regulation of (K) *FABP1* and (L) *Serpina1* gene expression.

Activin B upregulate sonic hedgehog signaling

To explore the potential effect of Activin A and B on *Shh* signaling we analyzed the gene expression of *Shh* and the Shh target gene *Gli1* in EBs cultured for 20 days and exposed to Activin A, Activin B and Activin B + SB431542. The present results demonstrate that *Shh* as well as *Gli1* gene expression is markedly up-regulated by Activin B as compared to control and Activin A treated cultures, and *Shh* is expressed in the area where *Pdx1* expression is observed (Fig. 4).

Discussion

The present study examined the effect of Activin A and Activin B on *pdx1* expression in hESCs differentiated as embryoid bodies (EBs). The results demonstrate that Activin B is a more potent inducer of *pdx1* gene and protein expression as compared to Activin A and untreated control cultures. Using human fetal and embryonic tissues it is demonstrated that the pancreas anlage in humans can be identified by the co-expression of *pdx1* and *nkx6.1*. Co-expression of *pdx-1* and *nkx6.1* was, however, not observed in Activin B treated EBs, thus suggesting that the *pdx1* expressing cells was not committed to a pancreatic fate at this stage of EB development. The lack of pancreas commitment may be explained by the present finding that Activin B, which in contrast to work in chick embryos, up-regulate the expression of *Shh* and its target *Gli1*. *Shh* signaling is normally permissive for liver and intestinal development and indeed *pdx1* expressing cells were observed in epithelial cell clusters also expressing proteins associated with early liver and intestinal development.

Activin signaling has previously been implicated in *Pdx1* and *Shh* induction, although, the precise role of the different Activins is controversial and not well established. Work in chick embryos showed that *Pdx1* specification requires notochord-mediated repression of *Shh* in the pre-pancreatic endoderm and that this repressive activity can be mimicked by Activin B [8]. It has also been demonstrated that Activin A signaling is sufficient to induce *Pdx1* expression in chick endoderm [24]. *Pdx1* is, however, not only a pancreatic marker, but also a duodenal marker [21,22], and work on mouse embryos have demonstrated that Activin A induce the conversion of committed pancreatic endoderm to intestinal-like structures expressing *Pdx1* as well as *FABP* [25]. The Activin A mediated intestinal differentiation was blocked by cylopamine, and mimicked by Shh, whereas Activin B induced neither Shh nor intestinal differentiation [25]. Thus Activin signaling elicit opposite effects on Shh expression *in vivo* and it is possible that this depend on the ligand, target tissue and developmental stage. The present study shows that Activin B specify epithelial structures expressing *Pdx1*, *Shh* as well as *FABP*, whereas Activin A support self-renewal mechanisms in hESCs differentiated as EBs. The discrepancy between the present

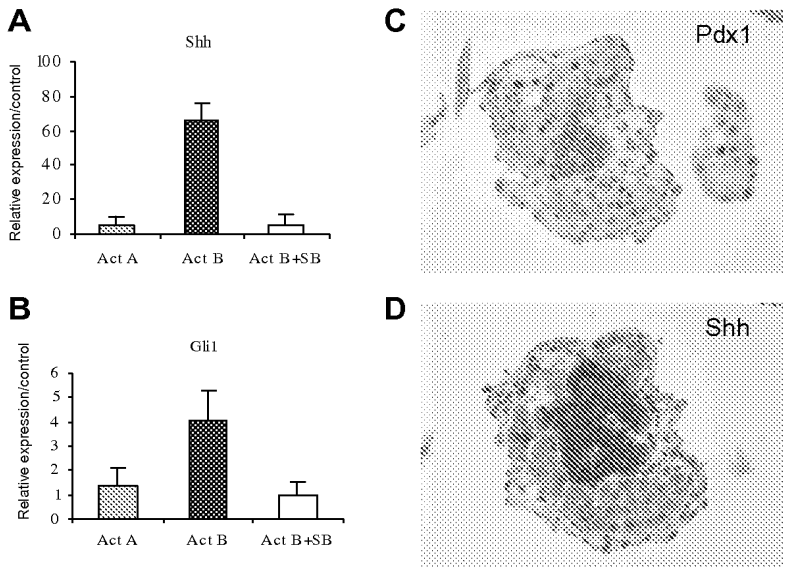

Fig. 4. Real-time PCR analysis for (A) sonic hedgehog (*shh*) and (B) the *Shh* target gene *Gli1* in EBs treated with (A) 50 ng/ml Activin A (Act A), 50 ng/ml) Activin B (Act B) and 50 ng/ml Activin B + 10 uM SB531542 (Act B + SB). The data show that *Shh* and *Gli1* gene expression is markedly up-regulated in EBs treated with Activin B. Immune histochemical stainings for (C) *Shh* and (D) *Pdx1* on serial sections of EB treated with Activin B for 20 days. The data show that Shh is expressed in the area where *Pdx1* expression is observed.

data and previous work with chick and mouse embryos is not clear. Nevertheless, the present data does support the idea that *Shh* expression in a limited territory of the endoderm permit intestinal/liver development whereas *Shh* repression in the adjacent dorsal and ventral regions is required for the development of pancreatic tissue [8,26]. Consequently, the present data suggest that Hedgehog repression at early differentiation stages is required for proper development of insulin-secreting cells from hESCs. This notion is also supported by recent work with mouse ES cells showing that Hedgehog production in EBs limits pancreatic fate acquisition [27]. As to Activin A, it has previously been demonstrated that Activin A is necessary and sufficient for the maintenance of self-renewal and pluripotency of hESCs and supports long-term feeder free growth of hESCs [28–30] and these findings support the current data demonstrating that *Oct-4*, *Nodal* and *Cripto* expression is maintained with Activin A treatment. Work with mouse and human embryonic stem cells has also demonstrated that Activin/nodal signaling is required for specification of definitive endoderm [12–15,17]. The generation of definitive endoderm from hESC requires, however, not only Activin/nodal signaling but also serum deprivation whereby the lack of insulin/IGF mediate suppression of phosphatidyllinositol 3-kinase signaling [31]. In the present study the differentiation media contained 15% serum replacement, thus explaining why activation of self-renewal programs was maintained over differentiation programs in response to Activin A.

An important finding in the present study was that *Pdx1* is broadly expressed in the human pancreas and duodenum during development and that the pancreas anlage can be identified by the co-expression of *Pdx1* and *nkx6.1*. These data demonstrate that *Pdx1* can not be used alone as a marker for pancreas progenitor cells in hESC differentiation studies. In summary, Activin B is a potent inducer of *Pdx1* as well as *Shh* in differentiating hESCs. The data suggest that additional suppression of *Shh* signaling may be required to allow for proper specification of pancreatic cell lineages in hESCs.

Acknowledgment

The authors wish to thank Raphael Scharfman and Ole D. Madsen for their respective gifts of the anti-*Pdx1* and anti-*nkx6.1* antibodies.

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